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Homolog to Virus Entry and Direct Cell-to-Cell Spread

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Experiments to analyze the functions of the equine herpesvirus 1 (EHV-1) glycoprotein gB were performed. Cell lines which stably expressed either the full-length EHV-1 gB or only the extracellular portion of gB (amino acids 1 to 844) were constructed and were termed TCgBf and TCgBΔ, respectively. Using the cell line TCgBf, a gB-negative viral mutant, L11ΔgB, was generated by replacing a 2.1-kb *Bgl*II–*Nru*I fragment in the EHV-1 strain RacL11 gB with the *Escherichia coli LacZ* gene. EHV-1 strain RacL11, the modified live vaccine strain RacH, and L11ΔgB were used for functional studies. It was shown that: (i) EHV-1 gB is essential for virus growth *in vitro* since gB-negative L11ΔgB exhibited titers of $<$10 PFU/ml when grown and titrated on noncomplementing cells. (ii) The cell line expressing truncated gB (TCgBΔ) did not complement for the growth of L11ΔgB, but the RacH virus grew to titers comparable to those of RacL11 in all cell lines tested. Since RacH had amino acids 944–980 of gB replaced by 7 missense amino acids as determined by nucleotide sequence analysis, the extreme carboxyterminus but not a domain between amino acid residues 845 and 943, probably the transmembrane domain, of EHV-1 gB is dispensable for virus growth in cultured cells. (iii) Single infected cells but no plaque formation were observed after infection of noncomplementing cells with L11ΔgB, demonstrating the requirement of EHV-1 gB for direct cell-to-cell spread of infection. (iv) The attachment of gB-negative L11ΔgB virions to target cells was similar to both phenotypically complemented L11ΔgB and parent RacL11 virus. (v) L11ΔgB viral titers could be enhanced by using the fusogen polyethylene glycol (PEG). The increase of L11ΔgB titers by PEG treatment, however, was considerably lower compared to gB-negative pseudorabies virus, suggesting that EHV-1 gB might not be as stringently required for virus penetration as are its homologs in other *Alphaherpesvirinae*.

INTRODUCTION

Equine herpesvirus 1 (EHV-1), a member of the *Alphaherpesvirinae,* is the causative agent of respiratory disease, abortions, and neurological disorders in horses (Allen and Bryans, 1986; O'Callaghan *et al.,* 1983). The entire genome of EHV-1 has been sequenced (Telford *et al.,* 1992) and genes encoding for glycoproteins which are homologous to the herpes simplex virus type 1 (HSV-1) glycoproteins gB through gE and gG through gM have been identified (Allen and Coogle, 1988; Colle *et al.,* 1992; Flowers and O'Callaghan, 1992; Flowers *et al.,* 1995; Pilling *et al.,* 1994; Robertson *et al.,* 1991; Telford *et al.,* 1992; Whalley *et al.,* 1989; Zhao *et al.,* 1992). Previously, six major EHV-1 envelope glycoproteins had been mapped using monoclonal antibodies and a λgt11-expression library (Allen and Yeargan, 1987). Nucleotide sequence and transcriptional analyses revealed that one of these major glycoproteins, gp14, represents the HSV-1 gB-homologous protein (Whalley *et al.,* 1989). Analyses of gp14 (referred to as EHV-1 gB in this communication) have shown that the glycoprotein is regulated as a $\beta-\gamma$ class molecule and that an M_r 118,000 primary translation product is N-glycosylated to yield an M_r 138,000 protein (Sullivan *et al.,* 1989). This glycosylated precursor molecule is cleaved and the two subunits form disulfide-bonded dimers both in infected cells and in mature virions (Meredith *et al.,* 1989; Sullivan *et al.,* 1989).

HSV-1 gB is essential for virus growth and involved both in virus penetration and in fusion of neighboring cells (Cai *et al.,* 1988a; Highlander *et al.,* 1988; Navarro *et al.,* 1992; Sarmiento *et al.,* 1979; Spear, 1993). In addition, studies applying anti-gB monoclonal antibodies and various HSV-1 gB mutants have defined domains which are involved in correct protein processing, penetration, cell-to-cell spread, and syncytium formation (Baghian *et al.,* 1993; Bzik *et al.,* 1984; Cai *et al.,* 1988a,b; DeLuca *et al.,* 1982; Gage *et al.,* 1993). Homologs of HSV-1 gB have been identified in all herpesviral subfamilies and the gB homologs of pseudorabies virus (PrV) and bovine herpesvirus 1 (BHV-1) display similar functions compared to those of HSV-1 gB and are also essential for virus growth

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(Kopp *et al.,* 1994; Miethke *et al.,* 1995; Peeters *et al.,* 1992; Rauh and Mettenleiter, 1991). Analyses of the gB-homologous proteins in HSV-1, PrV, and BHV-1 have shown that the cleavage of the gB precursor molecule is not essential for BHV-1 propagation but influences plaque size (Kopp *et al.,* 1994). The function of PrV gB can be complemented by BHV-1 gB, but not by HSV-1 gB, and the HSV-1 gB functions can be substituted by the PrV homolog. In contrast, BHV-1 gB functions cannot be replaced by PrV gB (Miethke *et al.,* 1995; Mettenleiter and Spear, 1994).

The EHV-1 strain Rac and the plaque isolates derived from different cell culture passages have been described previously. It has been demonstrated that the modified live vaccine strain RacH displays a deletion affecting the IR6 gene, and that RacH lacks a *Sal*I restriction site mapping to the gB gene. RacH is apathogenic for both the natural host and laboratory animals, whereas the low passage derivative RacL11 causes disease in horses, Syrian hamsters, and mice (Hübert *et al.,* 1996; Mayr *et al.,* 1968; Osterrieder *et al.,* 1995).

The aim of this study was to analyze the functions of the EHV-1 gB homolog. By using different EHV-1 gB-expressing cell lines, a gB-negative EHV-1 mutant, and the EHV-1 strains RacL11 and RacH it could be demonstrated that (i) EHV-1 gB is essential for direct cell-to-cell spread of virions, (ii) EHV-1 gB is not significantly impaired in attachment to target cells, (iii) it appears not as stringently required for virus penetration as the gB homologs are in other *Alphaherpesvirinae,* and (iv) the carboxyterminal 37 amino acids of EHV-1 gB are dispensable for virus growth in cell culture.

MATERIALS AND METHODS

Viruses and cells

EHV-1 strains RacL11 and RacH (Hübert *et al.,* 1996; Osterrieder *et al.,* 1996a) were propagated and titrated on Rk_{13} cells or the equine dermal cell line Edmin337, all maintained in MEM containing 5–10% fetal calf serum (FCS). EHV-1 virions were purified by centrifugation for 3 hr at 20° on 20–70% sucrose gradients in an SW40 rotor (30,000 rpm; Beckman) (Osterrieder *et al.,* 1996a). For counting physical virus particles, cells were infected and virions were harvested at 36 hr p.i. Cellular debris was removed by low-speed centrifugation (3000 *g,* 5 min) and supernatants (1 ml) were layered onto a 30% sucrose cushion. Virions were partially purified by ultracentrifugation (SW60 rotor, 40,000 rpm, 1 hr, 4°) and resuspended in 100 μl phosphate-buffered saline (PBS). These preparations were mixed with an equal volume of latex particles (330 nm; Plano, Wetzlar, Germany) at a concentration of 5.1×10^7/ml and centrifuged on copper grids using a Beckman airfuge (80,000 *g,* 20 min, 20°). Virions and latex particles on 10 grid sections were counted in an electron microscope and the number of virus particles was determined by the equation $n = 2.55 \times 10^6 \times V/L$ where n is the number of virus particles per milliliter, V is the number of counted intact virions, and L is the number of counted latex particles.

Plasmids

For nucleotide sequence analyses, a 5.1-kb *Pst*I fragment of EHV-1 strains RacL11 and RacH was cloned into pTZ18R (Pharmacia) resulting in plasmids pLP5 and p06, respectively. Further, the entire gB open reading frame of RacL11 was amplified by a standard PCR (Saiki *et al.,* 1988) using the thermostable *Pfu* polymerase (Stratagene) and conventional primers (5′-primer, 5′-ACGTGAATTCATGTCCTCTGGTTGCCG-3′; 3′-primer, 5′-TAAATATCCTTGGGGCCG-3′). The resulting 3.0-kb amplicon was cloned into pcDNAI/Amp (Invitrogen) and the recombinant plasmid was termed pCB3.0. Plasmid pDES1 containing the truncated RacH gB-gene (aa 1 to 844) under the control of the CMV promoter has been described previously (Osterrieder *et al.,* 1995) For the construction of pgBβ^+N, a 2.1-kb *Bgl*II–*Nru*I fragment located within the gB gene was removed from plasmid p06 (removing aa 220 to 921 of EHV-1 gB) to introduce the 4-kb *LacZ* expression cassette contained in pTT264A$^+$ (Mettenleiter and Rauh, 1990; kindly provided by W. Fuchs and T. C. Mettenleiter). The cassette was released with *Bam*HI and *Sal*I, and blunt-end ligated after fill-in of 5′-overhangs with the Klenow polymerase (Sambrook *et al.,* 1989). The location and construction of the plasmids are depicted in Fig. 1.

Construction of gB-expressing cell lines and a gB-negative EHV-1

Rk_{13} cells (10^5 cells) were cotransfected by the calcium phosphate precipitation method with 1 to 10 μg of pCB3.0 or pDES1 and 1 μg of pSV2-neo exactly as previously described (Osterrieder *et al.,* 1996b). Transfected cells were grown in TC199 medium containing 10% FCS and 800 μg/ml G418 (Gibco BRL) and cloned by limiting dilution. The cell line expressing full-length EHV-1 gB was termed TCgBf and the cell line expressing amino acids 1 to 844 of gB was termed TCgBΔ. The cell line TCgBf was used for the construction of gB-negative EHV-1 which were obtained by cotransfection of RacL11 DNA (0.5–2 μg) with varying amounts (1–10 μg) of pgBβ^+N by lipotransfection using the DOTAP reagent (Boehringer).

DNA analyses

Viral DNA was prepared from purified virions. Restriction enzyme digests of viral DNA were separated on 0.8% agarose gels and transferred to positively charged nylon membranes (HybondN$^+$; Amersham) by vacuum blotting using 0.4 *M* NaOH (Reed and Mann, 1985). The 3.0-kb

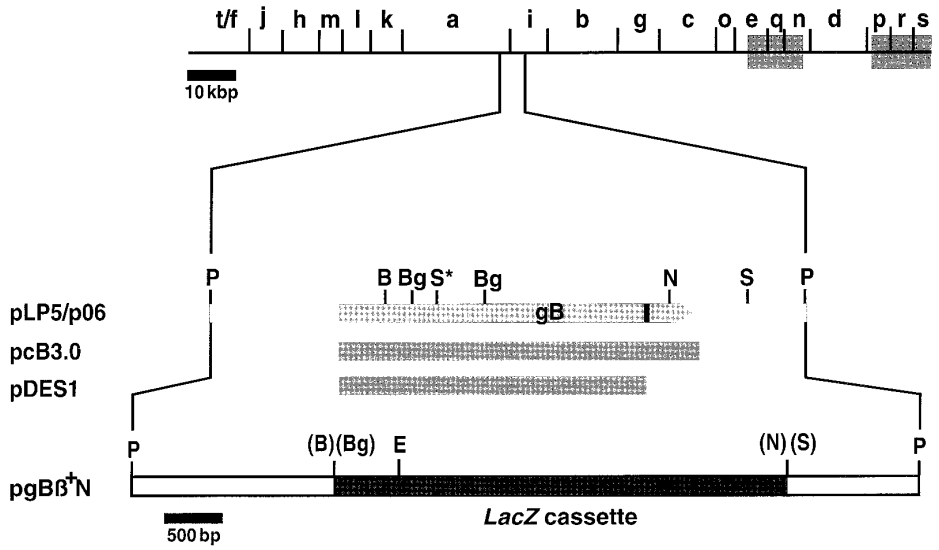

FIG. 1. Schematic illustration of the generated plasmids. Shown is the *Bam*HI map of EHV-1 strain RacL11 and the location of the EHV-1 gB open reading frame. The open reading frame is shaded and the transmembrane domain is indicated by a black vertical bar. Plasmids pLP5 (RacL11) and p06 (RacH) as well as the expression plasmids pCB3.0 containing the full-length EHV-1 gB and pDES1 containing aa 1–844 of gB are depicted. The plasmid pgBβ^+N containing the *Escherichia coli LacZ* gene was constructed as described under Material and Methods. Restriction sites are given: B, *Bam*HI; Bg, *Bgl*II; P, *Pst*I; S, *Sal*I; E, *Eco*RI; N, *Nru*I. The *Sal*I site in the RacL11 gB open reading frame that is absent in RacH gB is indicated by an asterisk (S*).

fragment contained in pCB3.0 and the 4.0-kb fragment contained in pTT264A$^+$ (Fig. 1) were labeled with digoxigenin-11-dUTP (Boehringer) after random priming and used as gB- and *LacZ*-specific probes, respectively. Sheets were incubated with labeled and heat-denatured probes suspended in hybridization solution (Sambrook *et al.,* 1989). Detection of DNA–DNA hybrids with an anti-digoxigenin AP conjugate and subsequent chemiluminescence with CDP-Star were performed according to the manufacturer's instructions (Boehringer). DNA sequences of RacL11 and RacH gB were determined by cycle sequencing of plasmids pLP5 and p06 using generated primers which were specific for EHV-1 gB (Table 1) and by an automatic DNA sequencer (Applied Biosystems). The sequences reported here were submitted to the EMBL Databank and have been assigned the Accession Nos. X95374 and X95377.

Antibodies

The anti-gB monoclonal antibodies used were 3F6 (kindly provided by G. P. Allen, Lexington, KY), 4B6, and Be/3F8. Mab 3F6 recognizes a linear epitope located between aa 107 and 171 of EHV-1 gB (Guo *et al.,* 1990), and mab 4B6 also binds to the aminoterminal subunit of gB (Osterrieder *et al.,* 1995). Mab Be/3F8 recognizes the carboxyterminal subunit of gB (Huemer *et al.,* 1995). Rabbit polyclonal antisera against gD (Flowers *et al.,* 1995) or IR6 (O'Callaghan *et al.,* 1994) (both rabbit antisera were kindly provided by D. J. O'Callaghan, Shreveport, LA), as well as anti-gM mab 13B2 (Allen and Yeargan, 1987; kindly provided by G. P. Allen) were used as control antibodies.

Indirect immunofluorescence and Western blot analyses

Detection of viral proteins in infected cells or the cell lines TCgBf and TCgBΔ by indirect immunofluorescence staining and subsequent flow cytometry (FACscan; Becton–Dickinson) was done exactly as described previously (Osterrieder *et al.,* 1995, 1996b). For SDS–10% polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970), lysates of purified virions, infected and mock-infected cells, or TCgBf and TCgBΔ cell lines were adjusted to a protein concentration of 5 mg/ml using a colorimetric test (BCA protein assay; Pierce) and suspended in sample buffer (Sambrook *et al.,* 1989) with or without addition of 5% 2-mercaptoethanol (2-ME). Cell culture supernatants of TCgBf and TCgBΔ cells were harvested and concentrated (50-fold) after addition of an equal volume of 4 *M* $(NH_4)_2SO_4$ by centrifugation, and sample buffer was added. Separated proteins were transferred to nitrocellulose by the semidry method (Kyhse-Andersen, 1984) and free binding sites were blocked with 10% nonfat dry milk in PBS containing 0.05% Tween 20 (Sigma). Detection with the respective antibodies followed, and bound antibodies were visualized with anti-mouse or anti-rabbit IgG alkaline phospatase conjugate (Sigma) and color reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

TABLE 1

Primers Used for Cycle Sequencing of gB Genes of RacL11 and RacH

Positive-strand primers		Negative-strand primers	
Position[a]	Sequence	Position	Sequence
886	5′-ACCTACCCGGACTCCAGC-3′	1147	5′-TAGAACTATTGTGCCCACCC-3′
1137	5′-GCATCATCGAGGGTGGGC-3′	1475	5′-TAGGGAGCGATGTTTTCC-3′
1394	5′-CGTACGCATCGAACCACC-3′	1802	5′-GTGTAGTGCCTCCATGGC-3′
1675	5′-TCATGCACCACGCGTACC-3′	2165	5′-TAAATCTGTAGGACCCGCGG-3′
1834	5′-ACTGCATCGTCGAGGAGG-3′	2441	5′-TTTTTAGGTCGTAGGTGCGG-3′
2080	5′-TCAGCTGGAACTGGACCG-3′	2711	5′-TCATTGACGTGGGATTGG-3′
2360	5′-CGGGGGGTTCTTAATTGC-3′	2947	5′-GGCGGGAGTAGCACGTGT-3′
2622	5′-TCTTCCAACGTCACCGCC-3′	3218	5′-TCCAGGGGCAGAAACTCG-3′
3010	5′-CGATAGAAGGCCAGCTGG-3′	3445	5′-TTGAAACAACAGCGCCGG-3′
3380	5′-CGCCAGCTTTTTCAAGAG-3′	3704	5′-TTGATCATTTCGCGAGCC-3′
3649	5′-ACGGCCAGAACGAGGAGG-3′	3964	5′-TAAATATCCTTGGGGCCG-3′

[a] The nucleotide position of the primers is indicated according to the sequence reported by Whalley *et al.,* 1989.

Radiolabeling of viral proteins and immunoprecipitation

Viral proteins were labeled using [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-Label; ICN). For production of radiolabeled virions, cells were infected with 5 PFU per cell and continuously labeled for 36 hr using methionine- and cysteine-free medium which was supplemented with 10% of the normal amount of methionine and 50 μCi/ml ^{35}S-labeled amino acids. For immunoprecipitations, infected cells were continuously labeled from 2 to 18 hr postinfection. Cells were lysed as described above and used for immunoprecipitations with mabs 3F6, Be/3F8, 4B6, and 13B2. Immunoprecipitations were performed with 2 μl of antibodies (containing approximately 5 mg/ml IgG) exactly as described by Flowers *et al.* (1995). Labeled proteins were separated by SDS–PAGE and analyzed by autoradiography.

Adsorption assays

Adsorption assays were performed by liquid scintillation counting using purified ^{35}S-labeled virions following the protocol described by Herold *et al.* (1991). Briefly, Rk_{13} and Edmin337 cells were seeded into 96-well plates (5000 cells per well). Cells were preincubated with PBS supplemented with 5% FCS for 1 hr. Radiolabeled virus (2.5×10^7 particles in 20 μl PBS + 5% FCS) was added to the cells and adsorption was allowed for 0.5 to 24 hr at 4°. Unbound virus was removed by five consecutive washes with PBS and the wash solutions were collected in scintillation vials. Cells were lysed with 1% sodium dodecyl sulfate to determine cell-associated radioactivity. Adsorption was calculated in percentages (cell-associated cpm/input cpm $\times$ 100%). At least four independent experiments were evaluated.

Chemical induction of penetration by treatment with polyethylene glycol (PEG)

Chemically induced fusion of viral envelopes with cellular membranes was achieved by following the protocol using the fusogen PEG as described by Sarmiento *et al.* (1979). Edmin337, Rk_{13} cells, or the cell lines TCgBf or TCgBΔ were incubated with 10-fold dilutions of RacL11 or L11ΔgB virus preparations for 1 hr at 37°. The medium was then removed, the cells were washed once with PBS, and a 1:1 PEG:MEM solution (made by mixing 40 g of melted PEG 6000 in 36.4 ml MEM) was added. The PEG solution was removed immediately and two washes followed (once with an 1:3 PEG:MEM solution and once with an 1:7 PEG:MEM solution). Monolayers were then washed three times with MEM containing 10% FCS. Control cells were treated accordingly but no PEG was present in the respective medium solutions. After the final washing step, MEM with 10% FCS was added and cells were incubated for 3 hr at 37° to allow fusion. Cells were then overlaid with methylcellulose, and plaques or single infected cells were counted 3 days after infection.

RESULTS

Sequence of RacL11 and RacH gB

The nucleotide sequences of RacL11 and RacH gB were determined by cycle sequencing of the recombinant plasmids pLP5 containing the entire RacL11 gB ORF and p06 (containing the RacH gB ORF). The use of the primers listed in Table 1 allowed the sequence determination of both DNA strands. The nucleotide sequences were subsequently analyzed using the GCG software package (Devereux *et al.,* 1984). The deduced RacL11 gB amino acid (aa) sequence exhibited a 99.6% homology to those of EHV-1 strains Ab4 and HVS25 (Telford *et al.,* 1992;

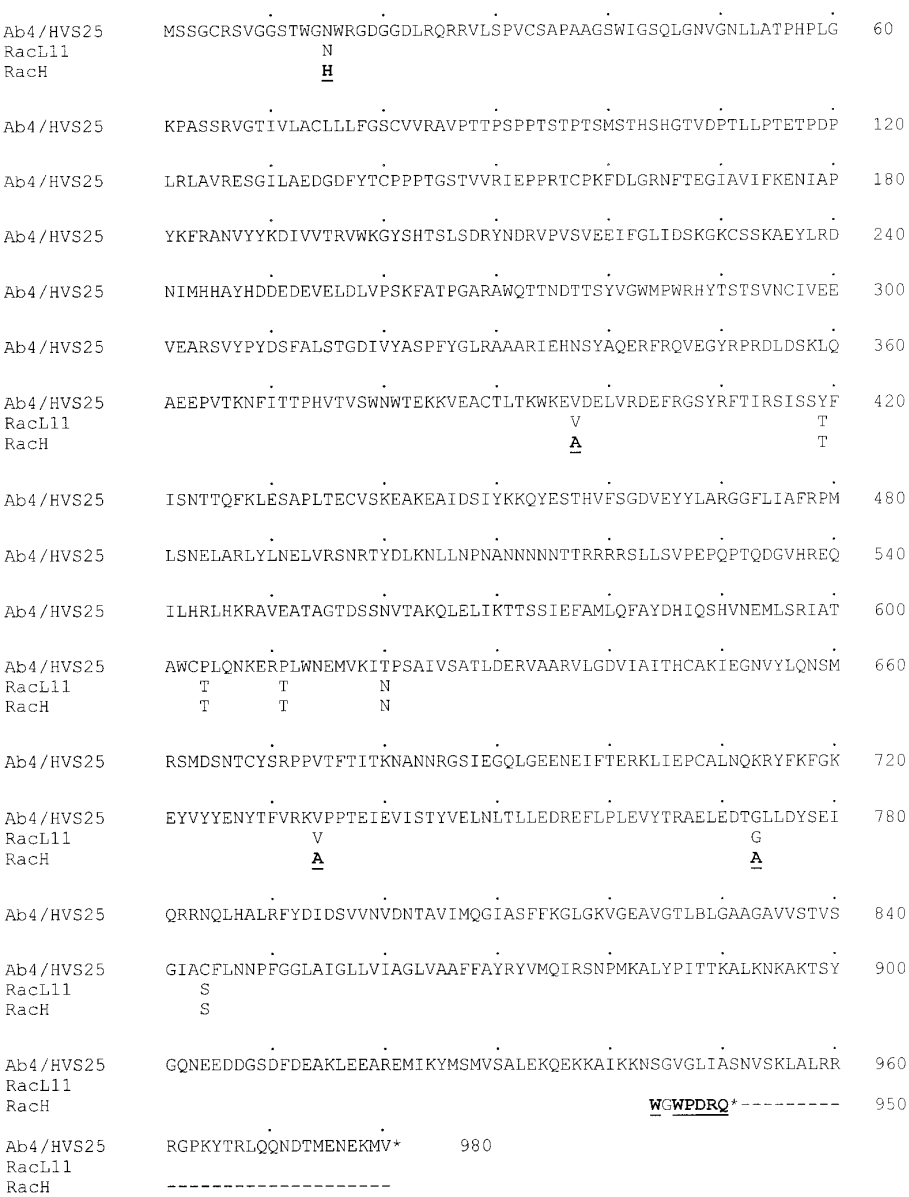

FIG. 2. Amino acid sequence of RacL11 and RacH gB compared to the gB sequences determined for strains Ab4p (Telford *et al.,* 1992) and HVS25 (Whalley *et al.,* 1989). Indicated are amino acid substitutions. The amino acids printed in bold and underlined are different between wild-type RacL11 and RacH. Also shown is the replacement of amino acids 944 to 980 by 7 missense amino acids in RacH gB. Stop codons are indicated by asterisks.

Whalley *et al.,* 1989) (Fig. 2). RacH gB displayed four aa exchanges compared to that of the pathogenic parental virus RacL11, i.e., replacement of Asp-14 by His, Val-397 and Val-734 by Ala, and Gly-773 by Ala. All substitutions are caused by nucleotide point mutations in the RacH gB nucleotide sequence. Of these point mutations, the Val-397 by Ala substitution concomitantly results in the loss of a *Sal*I site in the RacH genome as has been reported earlier (Hübert *et al.,* 1996). In addition, the carboxyterminal 37 aa of RacH gB are replaced by 7 missense amino acids (Fig. 2), which is due to a frameshift mutation following the deletion of 2 nucleotides (A and G) after nucleotide 2829 of the RacL11 gB nucleotide sequence. Western blot analyses using mabs 3F6, 4B6, and Be/3F8 demonstrated that both RacL11 and RacH were able to express gB in comparable amounts in infected cells, and that gB was incorporated in comparable amounts in purified virions of both viruses (Osterrieder *et al.,* 1996a) (data not shown).

Construction and characterization of cell lines expressing EHV-1 gB

The cell line TCgBf which constitutively expresses full-length RacL11 gB (aa 1 to 980) was constructed by co-

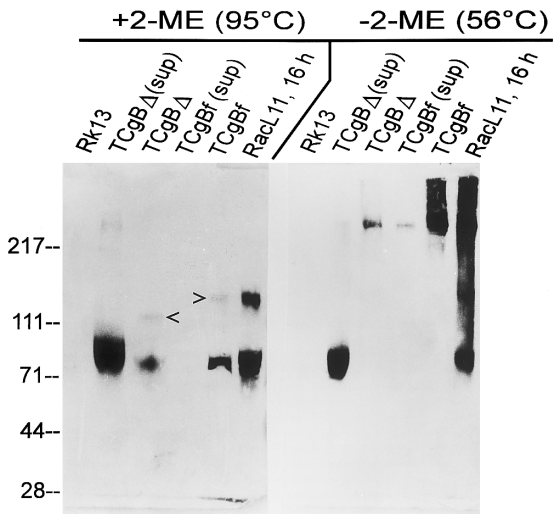

FIG. 3. Immunoblots of lysates from gB-expressing cell lines TCgBf and TCgBΔ as well as Rk_{13} cells and RacL11-infected cells which were used as controls. Cell lysates were prepared, suspended in sample buffer with (+2-ME) or without (−2-ME) 2-mercaptoethanol, incubated at the indicated temperatures for 3 min, and separated by SDS–10% PAGE. Supernatants of the cell lines were prepared as described under Materials and Methods and treated accordingly. After gel electrophoresis, the proteins were transferred to nitrocellulose and probed with anti-gB mab 3F6. Molecular weights are indicated in thousands and gB-precursor molecules in lysates of the TCgBf and TCgBΔ cell lines are marked by arrowheads.

transfection of plasmids pCB3.0 and pSV2-neo into Rk_{13} cells. Isolated G418-resistant cell clones were selected for expression of EHV-1 gB by indirect immunofluorescence analysis in a FACScan flow cytometer using the anti-EHV-1 gB mab 4B6. Accordingly, the cell line TCgBΔ expressing aa 1 to 844 of EHV-1 gB was isolated after cotransfection of pDES1 and pSV2-neo into Rk_{13} cells. In both cell lines, >95% of the cells were producing EHV-1 gB as analyzed by flow cytometry (data not shown). The cell lines were further analyzed by immunoblotting under different conditions using anti-EHV-1 gB mab 3F6. Cell lysates and cell culture supernatants of TCgBf and TCgBΔ were prepared, suspended in sample buffer with or without the addition of 2-ME, and separated by SDS–10% PAGE. In the presence of 2-ME and after heating of the samples to 95°, bands with apparent M_rs of 75,000–78,000 and 138,000 reacted with mab 3F6 in lysates of TCgBf. Thus, the reaction pattern of TCgBf cell lysates with mab 3F6 was very similar to that of RacL11-infected cell lysates prepared at 16 hr after infection (Fig. 3). No detectable reactivity with the 3F6 antibody was obtained when TCgBf cell culture supernatants were analyzed (Fig. 3). In TCgBΔ cell lysates, the same M_r 75,000–78,000 band as was in lysates of TCgBf cells or RacL11-infected cell lysates was detected. However, the uncleaved gB-precursor molecule was reduced in size and exhibited an apparent M_r of 120,000 (Fig. 3). In cell culture supernatants of TCgBΔ cells, an M_r 75,000–78,000 protein strongly reacted with mab 3F6 (Fig. 3), indicating the secretion of gB into the cell culture supernatant. When the same samples were prepared in the absence of 2-ME and heated to 56°, proteins with an M_r of approximately 250,000 were observed in all preparations, indicating an oligomerization of EHV-1 gB in both TCgBf and TCgBΔ cells; these oligomers were also observed in RacL11-infected cells under these conditions (Fig. 3). Some EHV-1 gB-specific reactivity was observed in supernatants of TCgBf cells, whereas a strong reaction of the 3F6 antibody with the M_r 75,000–78,000 gB band was detected in TCgBΔ cell supernatants. Only a faint reaction with the apparent M_r 250,000 band was observed in TCgBΔ supernatants under these conditions (Fig. 3). These data indicated that both subunits of EHV-1 gB were secreted into the supernatant of TCgBΔ cells, but that the oligomeric complex appears to be less stable in cell culture supernatants than within cells. Taken together, TCgBf and TCgBΔ cells were shown to express EHV-1 gB and the respective gB proteins appeared to be properly processed in that both the cleavage and the oligomeric structure of the molecule were retained. The fact that the gB precursor molecule in TCgBΔ cells was reduced in size and that the gB subunits are secreted into the cell culture supernatant was probably due to the absence of the transmembrane portion of EHV-1 gB produced by this cell line.

Construction and analysis of a gB-negative EHV-1

The cell line TCgBf was used to engineer RacL11 viruses which are devoid of an intact gB ORF. To this end, the *Escherichia coli LacZ* gene under the control of the PrV gG promoter (Mettenleiter and Rauh, 1990) was inserted into the gB gene by cotransfection of TCgBf cells with pgBβ^+N plasmid DNA and RacL11 genomic DNA (see Fig. 1 and Material and Methods). Virus progeny was plated under an agarose overlay which contained Bluo-Gal. Blue-staining virus plaques were picked, purified by six consecutive rounds of plaque purification, and the progeny virus was termed L11ΔgB. To confirm the correct insertion of *LacZ* sequences into the gB gene, viral DNA of RacL11 or L11ΔgB was prepared and cleaved with the restriction enzymes *Bam*HI or *Pst*I. DNA fragments were separated on 0.8% agarose gels, transferred to nylon membranes, and probed with digoxigenin-labeled EHV-1 gB or *LacZ* sequences prepared from plasmids pCB3.0 or pTT264A$^+$, respectively. As expected, fragments of 20.1-kb (*Bam*HI-A fragment) and 6.9-kb (*Bam*HI-I fragment) hybridized with pCB3.0 in RacL11 DNA after cleavage with *Bam*HI. In *Pst*I-digested RacL11 DNA, a 5.1-kb fragment hybridized with that probe (Fig. 4). No reactivity was obtained with RacL11 DNA after hybridization with the *LacZ*-specific probe (Fig. 4). In DNA of L11ΔgB cleaved with *Bam*HI,

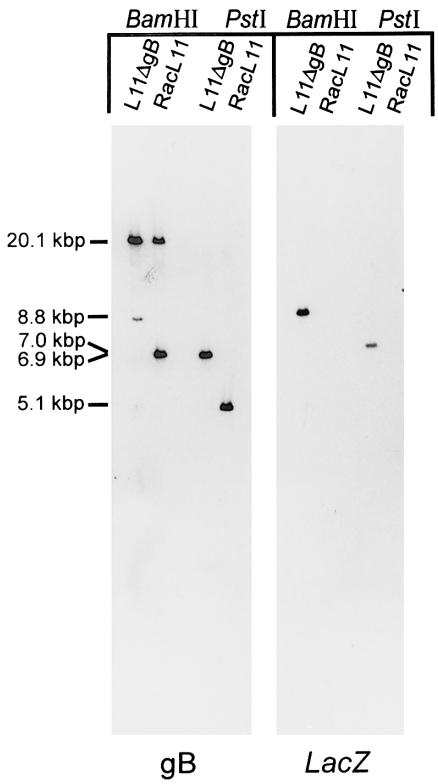

FIG. 4. Southern blot analysis of RacL11 and L11ΔgB DNA cleaved with the restriction enzymes *Bam*HI or *Pst*I and probed with a gB-specific probe prepared from plasmid pCB3.0 (gB) or a *LacZ*-specific probe derived from pTT264A$^+$ (*LacZ*). The sizes of the reactive fragments are indicated in kb.

fragments of 20.1 kb (*Bam*HI-A) and 8.8 kb in size reacted with the pCB3.0 probe (Fig. 4). Only the 8.8-kb *Bam*HI fragment in L11ΔgB DNA hybridized to the labeled *LacZ* probe (Fig. 4). In L11ΔgB DNA cleaved with *Pst*I, a 7.0-kb fragment was detected by both gB and *LacZ* sequences as probes (Fig. 4). From these data we concluded that the *LacZ* gene was correctly inserted into gB-encoding sequences.

As deduced from the nucleotide sequence of the EHV-1 gB–*LacZ* deletion construct, the expression of an aminoterminal 223-aa truncated gB polypeptide by L11ΔgB is theoretically possible. Anti-gB mab 3F6, which recognizes a linear epitope in this region (between aa 107 and 171; Guo *et al.,* 1990), mab 4B6, and mab Be/3F8 were used for immunoprecipitations to analyze ^{35}S-labeled virion polypeptides produced after infection (m.o.i. = 5) with RacL11 or L11ΔgB. After SDS–10% PAGE of the immunocomplexes, bands of M_r 138,000, 75,000 to 78,000, and 55,000, and also the high M_r form of EHV-1 gB (M_r 250,000) were detected after immunoprecipitations with all anti-gB antibodies in RacL11-infected cells. In contrast, no reactivity with either of the mabs was obtained with radiolabeled lysates of L11ΔgB-infected Rk_{13} cells (Fig. 5A). However, both in RacL11- and in L11ΔgB-infected cells, anti-gM mab 13B2 specifically immunoprecipitated gM (Fig. 5A).

The absence of gB expression in L11ΔgB-infected Rk_{13} cells in the presence of other gene products was confirmed by Western blot analysis using anti-gB mab 3F6, anti-gM mab 13B2, a monospecific anti-gD antiserum, and a monospecific antiserum directed against the early IR6 protein. Whereas in both RacL11- and L11ΔgB-infected cell lysates EHV-1 gD, EHV-1 gM, and the early IR6 protein were readily detectable at 24 hr p.i., the specific staining pattern with anti-gB mab 3F6 was only observed in RacL11-infected cell lysates and not in those of L11ΔgB (Fig. 5B).

To further ensure the absence of EHV-1 gB expression in L11ΔgB-infected cells, Rk_{13} cells infected with RacL11 or L11ΔgB were analyzed by indirect immunofluorescence using anti-gB mabs 4B6 and Be/3F8 as well as anti-gM mab 13B2. Cells were infected with 5 PFU per cell of RacL11 or L11ΔgB and harvested at 36 hr after infection. A specific staining of approximately 80% of the cells was observed with all antibodies in RacL11-infected cells (Fig. 6). In contrast, anti-gB mabs 4B6 and Be/3F8 did not react with L11ΔgB-infected Rk_{13} cells, whereas more than 70% of the cells strongly reacted with anti-gM mab 13B2 (Fig. 6).

These data confirmed that no detectable gB reactivity was obtained after infection of cells with L11ΔgB, but that infection occurred as reflected by the presence of the early IR6 as well as the late gM and gD gene products. Since neither the amino- nor the carboxyterminal subunit of EHV-1 gB was detected in L11ΔgB-infected Rk_{13} cells by anti-gB antibodies in three different tests, it was concluded that L11ΔgB is not capable of encoding for an intact or aberrant form of EHV-1 gB.

Growth of the L11ΔgB mutant on different host cells

To analyze the growth properties of the gB-negative L11ΔgB mutant, of the parental RacL11 virus, and of the RacH virus, gB-complementing cells (TCgBf), the cell line expressing truncated gB (TCgBΔ), and the cell lines Rk_{13} and Edmin337 were infected with the respective viruses. Results of subsequent titration experiments are summarized in Table 2. L11ΔgB virus, which was produced on complementing TCgBf cells and had gB incorporated in viral envelopes as shown by Western blot analysis (data not shown), produced plaques on complementing cells which stained blue in the presence of Bluo-Gal (Fig. 7) and exhibited titers which were comparable to that of wild-type RacL11 and those of RacH (Table 2). In contrast, viral titers of L11ΔgB were reduced by approximately 10^5-fold when virus was produced and titrated on TCgBΔ, Rk_{13}, or Edmin337 cells, but single blue-staining cells were observed in the presence of Bluo-Gal (Fig. 7). However, when L11ΔgB was produced on noncomplementing cells and titrated on TCgBf cells, titers of approx-

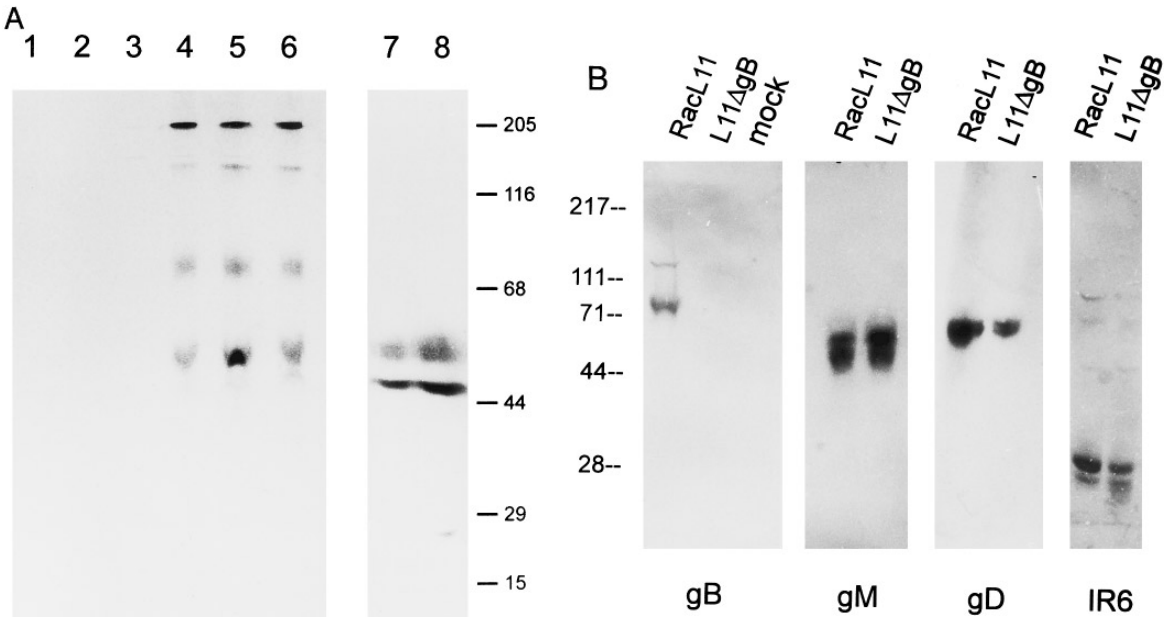

FIG. 5. (A) Immunoprecipitations of ^{35}S-labeled Rk_{13} cell lysates infected with L11ΔgB (lanes 1, 2, 3, and 7) or RacL11 (lanes 4, 5, 6, and 8) using anti-gB mabs 3F6 (lanes 1 and 4), 4B6 (lanes 2 and 5), and Be/3F8 (lanes 3 and 6) or mab 13B2 (lanes 7 and 8), which is directed against gM. Immunocomplexes were separated by SDS–10% PAGE and detected by autoradiography. Molecular weights are indicated in thousands. (B) Western blot analysis of RacL11- and L11ΔgB-infected cell lysates. Lysates of infected Rk_{13} cells (m.o.i. = 5) were harvested at 24 hr after infection, separated by SDS–10% PAGE, and transferred to nitrocellulose. Different sheets were incubated with anti-gB mab 3F6 (gB), anti-gM mab 13B2 (gM), or monospecific rabbit sera against gD (gD) and the early IR6 protein (IR6). Molecular weights are indicated in thousands.

imately 10^3/ml were observed indicating that virions phenotypically lacking gB — although with reduced efficiency — were also able to enter cells. The viral titers obtained with both RacL11 and RacH virus grown on TCgBf cells were virtually indistinguishable from each other on all cell lines (Table 2). These data indicated that: (i) gB is essential for EHV-1 growth, since gB-negative EHV-1 are able to produce virus plaques only on cells complementing for gB *in trans.* (ii) Phenotypically gB-complemented L11ΔgB is able to enter noncomplementing target cells but does not form plaques, demonstrating the essentiality of EHV-1 gB in direct cell-to-cell spread of virions. (iii) L11ΔgB virions phenotypically lacking gB are able to infect both complementing and noncomplementing cells although at an approximately 1000-fold reduced efficiency compared to wild-type RacL11. (iv) Since RacH, which had aa 944 to 980 replaced with 7 missense amino acids, grows on noncomplementing cells, this portion of gB is apparently not essential for virus growth in cultured cells. (v) The cell line TCgBΔ lacking the transmembrane domain of EHV-1 gB (aa 853 to 868; Whalley *et al.,* 1989) did not complement for the production of infectious virus progeny and plaque formation of L11ΔgB, suggesting that membrane anchoring of EHV-1 gB is essential for virus growth.

Specific infectivities of RacL11 and L11ΔgB

The titration experiments had demonstrated that L11ΔgB produced on noncomplementing cells was able to infect target cells, although at a largely reduced efficiency, as demonstrated by the production of viral plaques on complementing TCgBf cells and single infected cells on noncomplementing cells. To further characterize the efficiency of infection by the gB-negative EHV-1 mutant, the particle per PFU ratios of RacL11 and L11ΔgB produced on both complementing TCgBf and noncomplementing Rk_{13} cells were determined (Table 3). The mean particle per PFU ratio of L11ΔgB produced on Rk_{13} cells (6308) exceeded those of L11ΔgB produced on complementing cells (64) by 99-fold. Similarly, the RacL11 particle per PFU ratio (78) was exceeded by 81-fold by that of L11ΔgB produced on noncomplementing cells. The increased particle per PFU ratio of L11ΔgB produced on Rk_{13} cells compared to phenotypically complemented L11ΔgB or parent RacL11 indicated that gB-negative EHV-1 particles are impaired at a step early in infection.

Binding of L11ΔgB to target cells

To determine the binding of virions to target cells, Rk_{13} or Edmin337 cells were reacted with purified, radiolabeled RacL11 or L11ΔgB virions. L11ΔgB virions were produced on either complementing TCgBf cells or Rk_{13} cells. In pilot experiments, the binding kinetics of RacL11 and L11ΔgB virions produced on complementing and noncomplementing cells were determined. The experiments demonstrated that the equilibrium of virus binding

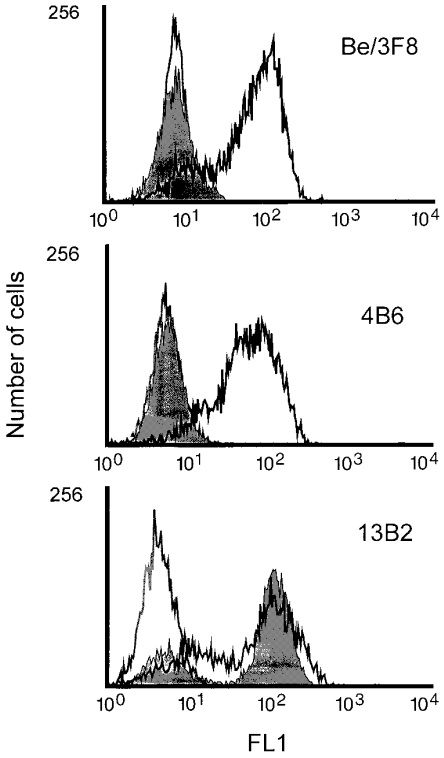

FIG. 6. Flow cytometric analyses of Rk_{13} cells. Cells were infected either with RacL11 or with L11ΔgB at an m.o.i. = 5, harvested at 36 hr p.i., fixed, permeabilized, and probed with anti-gB mabs 4B6 and Be/3F8 or anti-gM mab 13B2. The *x* axis shows the $\log_{10}$ fluorescence intensity 1 (FL1), and the *y* axis shows the number of cells. Gray-shaded curves represent L11ΔgB-infected cells, black solid lines represent RacL11-infected cells, and gray solid lines represent uninfected control cells.

was approached at 6 to 7 hr after infection where 18 to 23% of the input radioactivity remained associated with the cells. No major differences were observed in the kinetics or the total amount of binding of virions to either Rk_{13} or Edmin337 cells. Likewise, no significant differences in either the kinetics or the total binding of RacL11 or L11ΔgB to the target cells could be demonstrated. After a 2-hr adsorption period at 4°, 9.2 ± 0.7% of RacL11 virions, 8.9 ± 0.4% of L11ΔgB virions produced on complementing cells, and 8.0 ± 0.6% of L11ΔgB virions produced on noncomplementing cells had bound to Rk_{13} cells. Similar values were determined for the binding of purified virions to Edmin337 cells, in that 9.8 ± 1.1% (RacL11), 9.5 ± 0.9% (L11ΔgB produced on TCgBf cells), and 8.9 ± 0.5% (L11ΔgB produced on Rk_{13} cells) had bound to cells at this time point. From these results it was apparent that virions lacking gB were slightly impaired in binding to target cells, and that the reduced attachment of L11ΔgB that was not statistically significant was not responsible for the greatly increased particle per PFU ratio of gB-negative EHV-1.

PEG-induced infectivity of gB-negative L11ΔgB

The above-described experiments had shown that EHV-1 gB is absolutely essential for direct cell-to-cell spread of virions and that the specific infectivity of EHV-1 is greatly reduced in the absence of gB. However, the reduced specific infectivity of L11ΔgB was obviously not caused by a significantly impaired binding of gB-negative virions to target cells. The gB homologs of HSV-1, PrV, and BHV-1 are essential for penetration of virions into target cells (Rauh and Mettenleiter, 1991; Miethke *et al.,* 1995; Sarmiento *et al.,* 1979). To address the question whether L11ΔgB viral titers or the number of infected cells of L11ΔgB produced on noncomplementing cells could be increased by chemically inducing virus penetration, experiments were performed in which the fusogenic agent PEG was used for the fusion of viral and cellular membranes (Sarmiento *et al.,* 1979). To this end, both RacL11 and L11ΔgB virus were generated on TCgBf or Rk_{13} cells and titrated on these cell lines in the presence or absence of PEG. gB^- PrV (Rauh and

TABLE 2

Titers[a] of Wild-Type RacL11, RacH, and L11ΔgB on Different Host Cells

	TCgBf[b]			TCgBΔ[b]			Rk_{13}[b]			Edmin337[b]		
Cell line[c]	RacL11	RacH	L11ΔgB	RacL11	RacH	L11ΔgB	RacL11	RacH	L11ΔgB	RacL11	RacH	L11ΔgB
TCgBF	9.3×10^5	1.1×10^6	1.2×10^6	1.4×10^6	6.9×10^5	3.1×10^3	2.9×10^6	1.1×10^6	1.1×10^3	8.2×10^5	9.1×10^5	9.2×10^2
	2.5×10^6	9.7×10^5	8.5×10^5	9.8×10^5	1.3×10^6	9.7×10^2	7.7×10^5	9.2×10^5	2.3×10^3	1.3×10^6	1.3×10^6	2.2×10^3
TCgBΔ	9.7×10^5	8.1×10^5	$<10^d$	1.4×10^6	7.7×10^5	<10	2.2×10^6	1.0×10^6	<10	1.2×10^6	8.6×10^5	<10
	8.4×10^5	1.5×10^6	<10	2.1×10^6	1.9×10^6	<10	9.1×10^5	2.2×10^6	<10	2.9×10^6	9.6×10^5	<10
Rk_{13}	3.3×10^6	2.1×10^6	<10	9.3×10^5	8.4×10^5	<10	9.0×10^5	8.9×10^5	<10	9.0×10^5	9.7×10^5	<10
	1.4×10^6	9.4×10^5	<10	1.2×10^6	2.4×10^6	<10	1.1×10^6	1.3×10^6	<10	2.4×10^6	1.7×10^6	<10
Edmin337	2.8 ts 10^6	8.9×10^5	<10	1.7×10^6	8.5×10^5	<10	9.8×10^5	8.3×10^5	<10	2.1×10^6	7.9×10^5	<10
	2.4×10^6	2.7×10^6	<10	9.5×10^5	1.1×10^6	<10	8.4×10^5	1.9×10^6	<10	1.7×10^6	9.8×10^5	<10

[a] Titers were determined as the number of plaques counted after 5 days under a methylcellulose overlay.
[b] Cell lines on which progeny virus was produced.
[c] Cell lines on which virus progeny was titrated. The results of two independent experiments are given.
[d] No plaques were counted at the lowest dilution tested (10^{-1}).

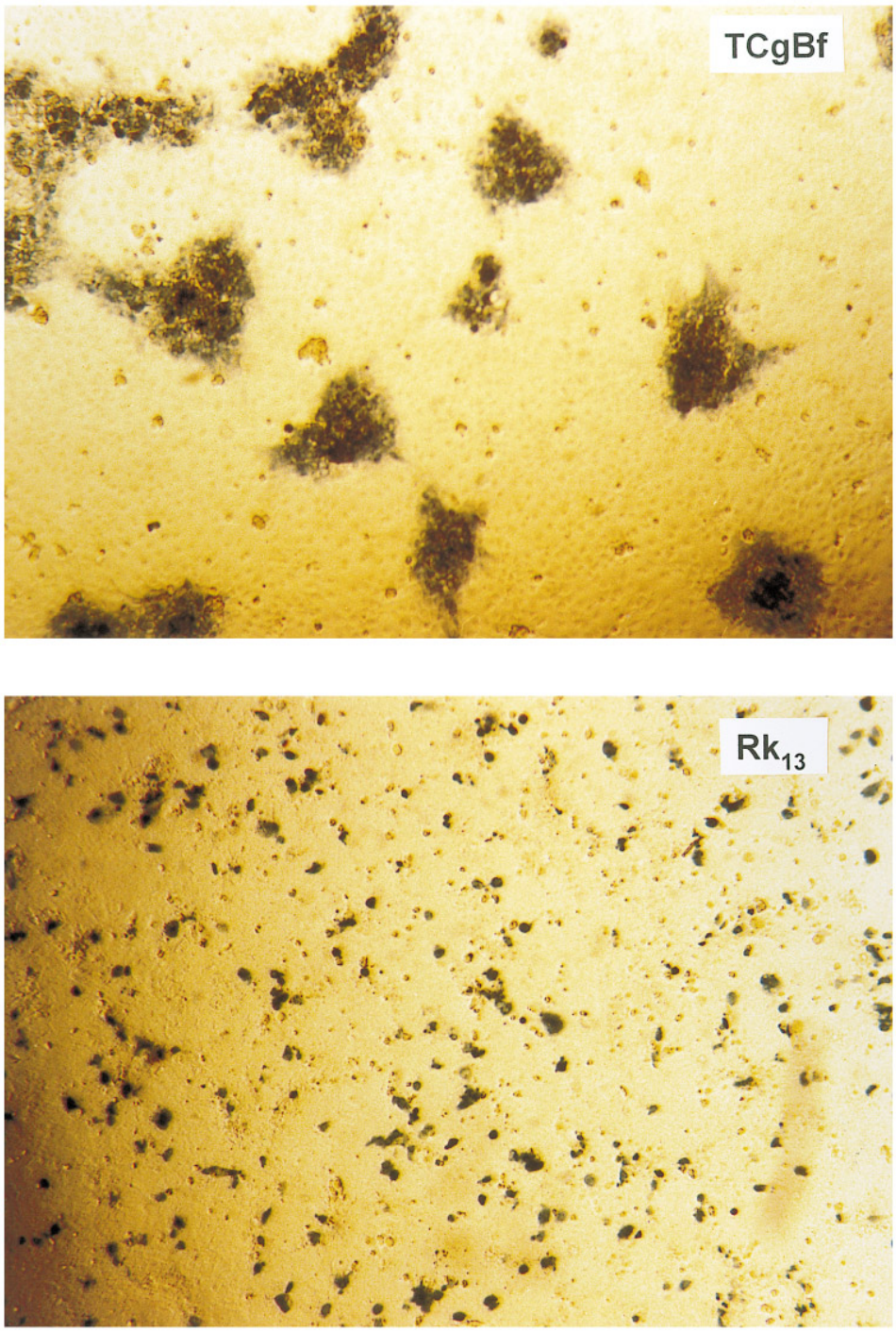

FIG. 7. Photographs of TCgBf and Rk_{13} cells infected with L11ΔgB. Cells were infected with the viruses and overlaid with methylcellulose containing 300 μg/ml Bluo-Gal. Three days after infection, cells were fixed with formalin and pictures were taken with a Leica camera on an inverted microscope. Whereas viral plaques were readily detected on TCgBf cells infected with L11ΔgB, only single blue-staining cells were observed after infection of Rk_{13} cells with L11ΔgB.

Mettenleiter, 1991; kindly provided by T. C. Mettenleiter, Insel Riems, Germany) was used as a control in these experiments. RacL11, gB-negative L11ΔgB, and gB^- PrV were produced by infecting the respective cells at an m.o.i. = 5. At 90 min after infection, infected cells were treated for 1 min with a citrate buffer (pH 3.0) to inactivate virus that had not entered cells and to avoid plaque formation by gB-complemented input virus in subsequent titration experiments (Highlander *et al.*, 1988). The produced virus preparations were titrated, cells were treated

TABLE 3

Particle/PFU Ratios of RacL11 and L11ΔgB Produced on Different Cell Lines

	RacL11 (Rk_{13})[a]			L11ΔgB (Rk_{13})[a]			L11ΔgB (TCgBf)[a]		
Expt[b]	Particles	PFU[c]	Ratio	Particles	PFU[c]	Ratio	Particles	PFU[c]	Ratio
1	2.8×10^8	2.7×10^6	104	8.2×10^6	2.3×10^3	3565	4.8×10^7	1.2×10^6	40
2	9.6×10^7	1.9×10^6	51	1.1×10^7	1.3×10^3	8461	1.4×10^8	2.2×10^6	64
3	1.1×10^8	1.4×10^6	79	6.9×10^6	1.0×10^3	6900	8.7×10^7	9.8×10^5	89
Mean[d]			78			6308			64

[a] The respective viruses were produced on the cell line indicated in brackets.
[b] Three independent experiments were performed.
[c] The plaque-forming units were determined by titration on Rk_{13} cells (RacL11) or TCgBf cells (L11ΔgB).
[d] The mean particle/PFU ratios from the three experiments are given.

with PEG, and virus-induced plaques or single infected cells obtained after treatment with the fusogen were counted and compared to those obtained on untreated cells. PEG treatment of cells after titration of RacL11 virus generally led to a slight reduction in virus titers both on gB-complementing and on noncomplementing cells (Table 4). L11ΔgB virus that phenotypically lacked gB exhibited virus titers which were increased in three independent experiments by 21- to 41.4-fold subsequent to PEG treatment of TCgBf cells (Table 4). Similarly, the number of infected cells which were observed for gB-negative L11ΔgB on noncomplementing Rk_{13} cells after PEG treatment was increased to nearly the same extent, i.e., from 15.1- to 22.2-fold (Table 4). The number of infected cells was determined by counting blue-staining cells after addition of the chromogen Bluo-Gal to fixed cells. In another series of experiments, L11ΔgB viruses were produced on the gB-complementing cell line TCgBf and titrated in the absence or presence of PEG. When TCgBf-produced L11ΔgB virus progeny was titrated on complementing cells, virtually no difference in virus titers with or without PEG treatment was observed, thus resembling the results obtained with wild-type RacL11 virus (Table 4). Similarly, the number of blue-staining cells observed after titration of phenotypically complemented L11ΔgB virus on Rk_{13} cells was slightly decreased after PEG treatment (ratios: 0.8 and 0.9; Table 4). In contrast, the titers of gB^- PrV could be enhanced by PEG treatment by 470- to 883-fold in three independent experiments (Table 4). Therefore, by chemically inducing membrane fusion with PEG, the titers of gB^- PrV were enhanced by 11- to 35-fold more than those of L11ΔgB. These results were confirmed by PEG experiments in which L11ΔgB and PrV gB^- were produced on noncomplementing cells without low pH treatment. Whereas titers of gB-negative L11ΔgB were increased by only 2.8-, 5.3-, and 7.5-fold after PEG treatment, those of gB^- PrV were increased by 69-, 94-, and 124-fold in three independent experiments. The results described here for an enhancement of gB^- PrV infectivity by PEG closely resembled those

TABLE 4

Influence of PEG on the Number of Plaques or Infected Cells

	RacL11 (Rk_{13})[a]			L11ΔgB (Rk_{13})[a,b]			L11ΔgB (TCgBf)[a,b]			PRV gII^- (Rk_{13})		
Cell line[c]	+PEG	−PEG	Ratio[d]	+PEG	−PEG	Ratio	+PEG	−PEG	Ratio	+PEG	−PEG	Ratio
TCgBf	3.2×10^6	2.7×10^6	1.2	9.1×10^4	2.2×10^3	41.4	1.3×10^6	1.2×10^6	1.1	9.4×10^3	2×10^1	470
	1.1×10^6	1.9×10^6	0.6	3.3×10^4	1.3×10^3	25.3	1.9×10^6	2.2×10^6	0.9	5.3×10^4	6×10^1	883
	8.8×10^5	1.4×10^6	0.6	2.1×10^4	1.0×10^3	21.0	8.3×10^5	9.8×10^5	0.8	3.7×10^4	6×10^1	617
Rk_{13}	8.1×10^5	9.8×10^5	0.8	9.1×10^3	4.1×10^3	22.2	1.1×10^6	1.2×10^6	0.9	ND	ND	
	9.1×10^5	2.2×10^6	0.4	1.1×10^4	7.3×10^2	15.1	7.9×10^5	1.1×10^6	0.8	ND	ND	
	7.4×10^5	1.0×10^6	0.7	3.9×10^4	2.6×10^3	19.5	ND[e]	ND		ND	ND	

[a] RacL11 or L11ΔgB were produced on the cell lines indicated.
[b] For L11ΔgB, the number of plaques (on TCgBf cells) or the number of single infected cells (noncomplementing Rk_{13} cells) was determined at Day 3 p.i.
[c] Cell lines on which virus progeny was titrated. The results of three independent experiments are given.
[d] Ratio of PFU or single infected cells with and without PEG treatment.
[e] Not determined.

reported earlier for gB$^-$ PrV using MDBK cells (Rauh and Mettenleiter, 1991). From the observations it was concluded that EHV-1 gB does not appear to be as stringently required for penetration into target cells compared to that of other *Alphaherpesvirinae,* since (i) virus titers of 10^3/ml were observed for L11ΔgB produced on noncomplementing cells, and since (ii) the increase in numbers of viral plaques or of single infected cells after artificial membrane fusion using PEG was significantly smaller for L11ΔgB than that determined for gB$^-$ PrV and those reported for gB-negative HSV-1 or BHV-1 mutants (Miethke *et al.,* 1995; Sarmiento *et al.,* 1979).

DISCUSSION

The presented study describes experiments which were conducted to elucidate the structure and functions of EHV-1 glycoprotein gB. The EHV-1 strain RacL11 and its high cell culture passage derivative RacH (Hübert *et al.,* 1996; Osterrieder *et al.,* 1996a), which express different gB molecules, and a gB-negative RacL11 virus (L11ΔgB) produced on a EHV-1 gB-complementing cell line were investigated.

The first series of experiments addressed the structure of both RacL11 and RacH gB. Nucleotide sequence analyses and the deduced aa sequences revealed a total of four aa substitutions in RacH gB compared to RacL11 gB and a replacement of the carboxyterminal 37 aa by 7 missense aa in RacH gB. Whereas the first aa substitution [His (RacH) for Asn] maps to the N-terminal signal sequence (Whalley *et al.,* 1989), the remaining three substitutions are located in the extracytoplasmic domain of the molecule. All changes represent conservative aa exchanges (Ala for Val, positions 397 and 734; Ala for Gly, position 773) and do not appear to influence the ability of the glycoprotein to aggregate to oligomeric structures as demonstrated by Western blot analyses. However, similar conservative aa exchanges were reported for a temperature-sensitive HSV-1 mutant (Bzik *et al.,* 1984) and truncations or mutations in the HSV-1 gB cytoplasmic tail as observed in RacH gB were associated with syncytium formation (Baghian *et al.,* 1993; Bzik *et al.,* 1984; Kousoulas *et al.,* 1984). Since RacH is avirulent for laboratory animals and the natural host (Hübert *et al.,* 1996; Mayr *et al.,* 1968), studies are in progress to analyze whether the mutations in RacH gB are involved in the more prominent syncytium formation of RacH on different cell lines (Neubauer *et al.,* unpublished) and also in the avirulence of RacH in animals by introducing RacH gB into L11ΔgB.

The second series of experiments reported in this study addressed the functions of EHV-1 gB. To this end, cell lines stably expressing different forms of gB and a gB-negative EHV-1 mutant (L11ΔgB) were constructed, and functional analyses were conducted. The experiments revealed that EHV-1 gB — like all other gB homologs analyzed to date — is essential for virus growth *in vitro* in that the L11ΔgB mutant virus could not be propagated on noncomplementing cells. Further analyses of the growth of RacH on noncomplementing cells as well as the growth of RacL11, RacH, and L11ΔgB on the cell line expressing truncated EHV-1 gB (TCgBΔ) showed that aa 944 to 980, but apparently not the transmembrane domain located between aa 853 to 868 of the polypeptide, are dispensable for virus growth.

The essentiality of EHV-1 gB for virus growth in cultured cells is primarily due to its absolute requirement for direct cell-to-cell spread of virions as shown by the absence of L11ΔgB virus plaques on noncomplementing cells. Virus binding assays demonstrated that EHV-1 gB, like its homologs in other *Alphaherpesviruses,* is not significantly involved in the overall binding of EHV-1 to the target cell. However, an elevated particle per PFU ratio of L11ΔgB compared to wild-type RacL11 indicated that gB-negative EHV-1 are impaired in an early step of infection. The involvement of EHV-1 gB in virus penetration was analyzed by artificially inducing membrane fusion by PEG. Using PEG, both increased numbers (up to 41.4-fold) of viral plaques on complementing cells and increased numbers of infected cells on noncomplementing cells were observed after plating of L11ΔgB produced in noncomplementing cells. However, the titers of gB$^-$ PrV which was used as a control in these experiments were enhanced by up to 883-fold. The absence of gB reactivity in L11ΔgB-infected cells and purified virions as assayed by Western blotting, flow cytometry, and immunoprecipitations suggests that EHV-1 gB is clearly involved in, but might not be as stringently required for, penetration, as is gB in other *Alphaherpesvirinae.*

The fact that virus penetration and viral cell-to-cell spread which both depend on fusion of membranes are related but distinct processes has been shown previously (Davis-Poynter *et al.,* 1994; Rauh and Mettenleiter, 1991). Hence, the findings reported here confirm and extend previous results on various homologs of herpesviral glycoproteins indicating that homologous glycoproteins (gB, gD, gM) may confer related but not strictly identical functions in different herpesviral systems (Baines and Roizman, 1993; Ligas and Johnson, 1988; Miethke *et al.,* 1995; Osterrieder *et al.,* 1996b; Rauh and Mettenleiter, 1991; Sarmiento *et al.,* 1979). To further elucidate the relationships of the gB homologs of different herpesviruses, a mutual replacement of the gB homologs has already been reported (Mettenleiter and Spear, 1994; Miethke *et al.,* 1995). The extension of these studies using EHV-1 gB might provide the possibility of gaining more insight into the mechanisms by which various gB homologs confer their functions in virus entry and direct cell-to-cell spread.

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